Human Vγ2Vδ2 T Cells Produce IFN-γ and TNF-α with an On/Off/On Cycling Pattern in Response to Live Bacterial Products[1]

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Whereas cytokine production in αβ T cells is rapidly regulated by exposure to peptide Ag, the mechanisms regulating cytokine production by γδ T cells are unknown. In this study, we demonstrate that human Vγ2Vδ2 T cells produce IFN-γ and TNF-α as early as 2 h after Ag exposure, and that they produce these cytokines in a dose- and time- dependent manner in response to stimulation with a live bacterial product, *iso*-butylamine (IBA), but not to dead bacteria or LPS. γδ T cells began, ceased, and then resumed IFN-γ and TNF-α generation in an on/off/on cycling pattern, both in vitro and in vivo, depending on the presence or absence of IBA. IFN-γ and TNF-α, whose optimum production was dependent on IBA-stimulated γδ T cells, were critical for monocyte-mediated killing of *Escherichia coli*. By limiting cytokine production to periods of direct contact with live bacteria, γδ T cells focus their resources at the site of infection, while limiting systemic immunopathology. Thus, human γδ T cells may mediate innate resistance to extracellular bacteria via tightly regulated cytokine production without necessarily expanding in number. *The Journal of Immunology*, 2001, 167: 6195–6201.

Found only in primates, human Vγ2Vδ2 T cells recognize nonpeptide organophosphate and alkylamine Ags secreted by bacteria in a TCR-dependent, and MHC- and CD1-unrestricted manner (1–3). The human pathogens *Morganella morganii* (1), *Salmonella typhimurium* (4, 5), and *Listeria monocytogenes* (6, 7) produce the antigenic alkylamines *iso*-butylamine (IBA),[3] *iso*-amylamine (IAA), and *n*-butylamine, respectively, and these bacterial infections cause in vivo γδ T cell expansions in humans. Other bacteria such as *Yersinia enterocolitica* and *Escherichia coli*, which produce the antigenic alkylamines IBA and IAA (4), cause the expansion of human γδ T cells in vitro (8, 9). *Clostridium perfringens* and *Bacteroides fragilis* secrete millimolar concentrations of *n*-butylamine and *n*-propylamine (10), which are antigenic alkylamines capable of expanding T cells in vitro (1). The parasite *Trichinella pseudospiralis*, which causes regressive and regenerative changes in muscle tissue, produces large amounts of *n*-butylamine (11).

γδ T cells expand 3–7 days after microbial infection (5, 9, 12, 13). However, the antibacterial effect of human γδ T cells in vivo is evident in as few as 17 h postinfection, indicating that expansion of γδ T cells is not required for an antibacterial response (36). Several lines of evidence suggest that γδ T cells participate in the immune response to microbial pathogens by producing factors such as IFN-γ and TNF-α, by direct cell-to-cell contact leading to cytotoxicity (14–16), or by producing granulysin and perforin (17). In animal models, one of dominant mechanisms for elimination of bacteria is dependent on monocyte-mediated killing, which is up-regulated by γδ T cell-derived IFN-γ (14, 15, 18).

γδ T cells comprise only 2–5% of $CD3^+$ cells in human peripheral blood, but are much more efficient cytokine producers on a per cell basis than αβ T cells (19). Release of IFN-γ and TNF-α can be harmful and sometimes lethal to the host (20–24). To maintain and mediate an effective immune response while minimizing systemic immunopathology, cytokine production should be precisely regulated. Production of IFN-γ and TNF-α in αβ T cells is rapidly regulated by exposure to peptide Ag (25). It is not known how human γδ T cells are regulated to produce those cytokines. Ideally, human γδ T cells should produce IFN-γ and TNF-α at a very early time in response to live bacterial products such as IBA or IAA, but not to dead bacteria or LPS. Once bacteria are killed, γδ T cells should cease cytokine generation to reduce and avoid unnecessary immunopathological reaction. Further, γδ T cells should quickly resume their cytokine production when bacterial infection occurs again. Polyclonal expansion of γδ T cells might be unnecessary in most circumstances such as in subclinical bacterial infections.

In this study, we demonstrate that human γδ T cells produce IFN-γ and TNF-α as early as 2 h after exposure to the live bacterial product IBA, a nonpeptide alkylamine Ag, but not to dead bacteria or LPS, in a dose- and time-dependent manner. γδ T cells began, ceased, and resumed IFN-γ and TNF-α generation in an on/off/on cycling pattern dependent on the presence or absence of IBA. IFN-γ and TNF-α, whose production was dependent on IBA-stimulated γδ T cells, were critical for monocyte-mediated killing of extracellular bacteria. Thus, by limiting cytokine production to periods of direct contact with live bacteria, γδ T cells focus their resources at the site of infection, while limiting systemic immunopathology.

Materials and Methods

Ab and Ag reagents

mAb ascites against T cell Ags used were as follows: control mAb (P3), pan-γδ TCR (anti-TCRδ1), Vδ1 (A13), Vδ1/Jδ1 (δTCS1), Vδ2 (BB3), Vγ2

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[3] Abbreviations used in this paper: IBA, *iso*-butylamine; IAA, *iso*-amylamine; LB, Luria-Bertani; BSS, balanced salt solution.

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(7A5), and CD3 (OKT3). The specificity of these mAbs is reviewed in Porcelli et al. (26). Other reagents were purchased as follows: FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (catalog number AMI4708; BioSource International, Camarillo, CA); IBA (catalog number I-3634; Sigma-Aldrich, St. Louis, MO); mouse anti-human IFN-γ (catalog number 554698; BD PharMingen, San Diego, CA); mouse anti-human TNF-α (catalog number 18630D; BD PharMingen); human TNF-α and IFN-γ (recDNA; National Cancer Institute, Bethesda, MD); and LPS (catalog L2880 from *E. coli* serotype 055:B5; Sigma-Aldrich).

PBMC

Human PBMC obtained from random healthy donor leukopacks (Dana-Farber Cancer Institute, Boston, MA) were isolated by Ficoll-Hypaque centrifugation (Pharmacia, Peapack, NJ). PBMC were screened for reactivity to Vγ2Vδ2 T cell Ags by culturing them at one million cells per well in 24-well flat-bottom plates in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 1 nM 2-ME, and 100 IU of penicillin and streptomycin in the presence or absence of IBA (0.4 mM) at 37°C. On day 3, IL-2 was added to a final concentration of 0.5 nM and on days 10–12, the cells were counted and analyzed by flow cytometry using TCR V gene-specific mAbs. Before expansion in culture, the percentage of Vδ2 T cells ranged from 1–5% of T cells. PBMC were considered positive responders to IBA if they showed a 5- to 40-fold expansion of Vγ2Vδ2 T cells (around 95% of screened donors) or produced intracellular IFN-γ and TNF-α (100% of screened donors). The screened PBMC were cryopreserved in FBS containing 10% DMSO at −196°C until use.

Depletion of Vγ2Vδ2 T cells

Depletion of Vγ2Vδ2 T cells was performed using mouse anti-human Vδ2 Ab (BB3), or P3, an isotype-matched mock control, and goat anti-mouse IgG Dynabeads M-450 (catalog number 110.06; Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. For most depletions, P3, an isotype-matched control mAb, was substituted for the anti-Vδ2 mAb. Over 95% of Vδ2 T cells, confirmed by surface marker staining and analysis of flow cytometry, were depleted. We screened several donors by two-color fluorescence and found that 100% of Vδ2-bearing T cells also expressed Vγ2. Though the Vδ2 TCR chain paired with Vγ1 or Vγ3 have been described, they are extremely rare, and there is no evidence that they respond to nonpeptide Ags. Thus, the likelihood that we are studying a population other than $V\gamma2V\delta2^+$ T cells is very remote.

Stimulation of PBMC with IBA, dead bacteria, or LPS in vitro

Fresh or cryopreserved PBMC were cultured in RPMI 1640 medium in the presence or absence of either 2 mM IBA, dead *E. coli* (25922; American Type Culture Collection, Manassas, VA; inactivated at 56°C for 2 h, and diluted to final concentration of 5×10^5 CFU/ml), or LPS (final concentration of 1 μg/ml). The culture supernatant at the indicated time points was collected for analysis of IFN-γ and TNF-α levels by ELISA, and PBMC were analyzed by three-color flow cytometry to quantitate percentage of Vγ2Vδ2 T cells producing IFN-γ and TNF-α.

Derivation of γδ T cell clones

PBMC were isolated from 40 ml of freshly collected peripheral blood by Ficoll-Hypaque centrifugation. One million PBMC were stimulated with alkylamine Ags in a 24-well plate using RPMI 1640 with 10% FBS. Fresh media containing 1 nM of rIL-2 was added on days 3 and 7. Flow cytometry performed on day 10 showed that γδ T cells expanded to 70–80% of $CD3^+$ cells. Culture was continued for another 2 wk. The cultured PBMC (1×10^7 cells) were reacted with the mAbs BMA031 and OKT4 to remove αβ T cells and $CD4^+$ T cells, respectively, by magnetic bead selection (27). The cells were cloned by limiting dilution in round-bottom 96-well plates in RPMI 1640 with 10% FBS, 1 nM rIL-2, and 1/2000 PHA-P. Irradiated (5 Gy) allogeneic PBMC (1×10^5) and EBV-transformed B cells (DG.EBV and CP.EBV each 2.5×10^4) were added to each well as feeder cells. The clones were maintained by periodic restimulation with PHA. T cells ($1–2 \times 10^5$/per well) were cultured with irradiated (5 Gy) allogeneic PBMC (5×10^5) and EBV-transformed B cells (DG.EBV and CP.EBV each 2.5×10^5) as feeders and PHA-P (1/4000) in RPMI 1640 supplemented with rIL-2 (1–2 nM) in 24-well plates.

Cytokine release assays

Stimulation of the γδ T cell clones was performed in a 96-well flat-bottom plate with 2×10^4 responder cells per well in 0.2 ml. Mitomycin C-treated C1R cells (5×10^4/per well) were used as accessory cells. Half log dilutions of IBA, IAA, or the calcium ionophore, ionomycin (at 1 μg/ml), plus 10 ng/ml PMA (as a positive control) were used (2). After a 24-h incubation, the supernatant was harvested and used at a final dilution of 1/8 to stimulate the growth of the IL-2-dependent HT-2 cell line. IL-2 release assays were performed in triplicate using 5×10^3 HT-2 cells per well in 96-well flat-bottom plates. After 18 h of culture, the HT-2 cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 24 h, and were then harvested and counted by liquid scintillation on a Betaplate system. The standard deviation of the triplicate determination was less then 10% of the mean. IFN-γ release was analyzed by ELISA (described below) after 24 h of antigenic stimulation.

Detection of IFN-γ in tissue culture supernatant

Human IFN-γ ELISA was performed according to procedures recommended by the manufacturer (catalog number 2613KI; BD PharMingen). The detection limit of the assay was 4.7 pg/ml.

Intracellular cytokine staining

PBMC were cultured in RPMI 1640 medium in presence or absence of IBA (1–2 mM), heat-killed *E. coli*, *M. morganii*, or *S. typhi* (1×10^7–1×10^8 CFU), or LPS (10 ng to 1 μg). Four hours before staining intracellular cytokine, monensin (catalog number 2092KZ; BD PharMingen) was added and cells were cultured at 37°C, which enhanced intracellular cytokine accumulation. Cells were washed with PBS and stained with surface marker AlexaFluor-conjugated 488 IgG control Ab or pan-TCRδ1 and $CD3^+$ (the Abs were purified and conjugated by our laboratory). After two washes, cells were fixed with 2% formaldehyde in PBS and permeabilized with 0.5% (w/v) saponin (catalog number 554715; BD PharMingen). Intracellular IFN-γ and TNF-α were stained with PE-conjugated Ab (catalog numbers 18905A and 18645A; BD PharMingen) in saponin buffer. After two washes, cells were resuspended in PBS and analyzed by use of FACS flow cytometer collecting 200,000 events per sample (BD Biosciences). The data were analyzed by Flowjo software (Tree Star, San Carlos, CA).

SCID mice

All animal experimental protocols were approved by the Internal Review Board of Brigham and Women's Hospital and Harvard Medical School. Homozygous C.B-17 *scid/scid* (SCID) male mice, 5–6 wk old, were purchased from Taconic Farms (Germantown, NY) and maintained in microisolator cages. Animals were fed autoclaved food and water. All manipulations were performed under laminar flow. One day before PBMC inoculation, SCID mice were injected (i.p.) with rabbit anti-asialo GM1 Ab (25 μl/mouse; WAKO, Richmond, VA), which depletes murine NK cells (28). SCID mice were irradiated (3 Gy gamma irradiation, ^{137}Cs source) before PBMC engraftment, which allows a high level of functional engraftment of human PBMC (29).

Treatment of PBMC with Vγ2Vδ2 specific Ags, engraftment of SCID mice, bacterial infection, and collection of engrafted PBMC

PBMC were cultivated in RPMI 1640 medium in the presence or absence of IBA (1 mM) for 18 h at 37°C, and all cells were collected and washed twice with RPMI 1640 medium. Groups of five SCID mice were injected with 0.5 ml (i.p.) of RPMI 1640 medium containing 3×10^7 human PBMC under aseptic conditions. *E. coli* (American Type Culture Collection 25922) was grown in Luria-Bertani (LB) broth at 37°C until the culture reached early stationary phase. *E. coli* was aliquoted (1 ml/vial) and stored in LB broth containing 10% glycerol at −80°C until use. Before infection, *E. coli* were washed once with 30 ml of PBS and plated on LB agar to determine CFU. Each SCID mouse was inoculated 0.5 ml (i.p.) PBS containing 5×10^6 CFU of live *E. coli*. Eighteen hours postinfection, PBMC were collected from the peritoneum with a 3-ml PBS lavage. The PBMC were washed three times with RPMI 1640 containing 100 IU/ml of penicillin and streptomycin, and 5 μg/ml of gentamicin. The intracellular cytokine staining was performed as described above.

Monocyte bactericidal assay (30)

After depletion or mock depletion of Vγ2Vδ2 T cells, human PBMC (1×10^6 cells in 1 ml of RPMI 1640-containing 1 mM IBA were cultivated in 24-well tissue culture plates for 4 days in the presence or absence of anti-IFN-γ (1 μg/ml), anti-TNF-α (1 μg/ml), IFN-γ (100 IU/ml), or TNF-α (100 IU/ml). The cells were washed three times with phosphate-buffered balanced salt solution (BSS) and resuspended in 0.65 ml of BSS. Fifty microliters of ice-cold normal human serum and 0.3 ml of *E. coli* (7.5×10^6 CFU/ml, middle stationary phase) were added to each well. After 20 min of cultivation at 37°C, cells were washed four to six times with 2 ml of ice-cold BSS (Sorvall RT 6000D, 1000 rpm, 8 min). One plate was taken

as time zero and Triton X-100 (1.5% in PBS) was added to release bacteria from the monocytes. Another plate was taken as time 90 min. Nine hundred-fifty μl of BSS and 50 μl of normal human serum was added to each well and was further incubated for 90 min at 37°C to allow for monocyte killing of bacteria before adding Triton X-100. Bacteria were diluted serially with water and was plated on LB agar and cultivated overnight at 37°C. The number of monocytes in wells treated with different conditions was the same, and the same number of bacteria was added for each condition (30). The range of bacterial counts after Triton X-100 treatment at the two time points was ~800 CFU at time 0, and 20–400 at time 90. The percentage of killing = [(test group T0 CFU − T90 CFU)/(mock-depletion T0 CFU − T90 CFU)] × 100.

Statistics

Values are expressed as means ± SEM of the respective test or control group. Statistical significance between control and test groups was calculated by the Student's *t* test (two-tailed) and among groups by analysis of variation. Data were representative of three to four experiments.

Results

Human γδ T cells produced IFN-γ and TNF-α in response to the live bacterial product, IBA, but not to dead bacteria and LPS

To determine which bacterial component was responsible for inducing cytokine production, we stimulated human γδ T cells with either IBA, heat-killed *E. coli*, heat-killed *M. morganii*, or LPS for 18 h, and we then analyzed intracellular IFN-γ and TNF-α production in γδ T cells using three-color flow cytometry. Over 1,000 Vγ2Vδ2 T cells in each sample, collected from 200,000 events of gated lymphocytes, were analyzed. The experiments were performed three times. None of the γδ T cells produced IFN-γ and TNF-α in response to dead bacteria and LPS, whereas 15.5% of γδ T cells generated IFN-γ and 9% generated TNF-α in response to 2 mM IBA, a live bacterial product (Fig. 1). IBA did not directly stimulate human αβ T cells, monocytes, NK cells, and NK T cells to produce IFN-γ and TNF-α, but LPS and dead bacteria did (data not shown). The dose of IBA used in this experiment is below the 3.4 mM of concentration detected in crude bacterial supernatant (1), suggesting that similar stimulation of γδ T cells might occur in vivo during infection. γδ T cells that responded to IBA stimulation and produced IFN-γ and TNF-α all expressed a TCR with markedly restricted germline gene segment usage (Vγ2Vδ2) in this study (data not shown).

Because dead bacteria and LPS up-regulate γδ T cells to express the activation markers CD25 and CD69 (16, 31), it is possible that they work synergistically with IBA in cytokine production. Interestingly, neither dead bacteria nor LPS augmented IBA-dependent cytokine secretion (Fig. 1), further demonstrating that γδ T cell-mediated production of IFN-γ and TNF-α was strictly regulated by the live bacterial product, IBA (Fig. 1).

Human Vγ2Vδ2 T cells produced IFN-γ and TNF-α as early as 2 h after Ag exposure, showing a time- and dose-dependent pattern

The prognosis of infection is dependent on the speed of immune system reaction and pathogen proliferation. Thus, early response to

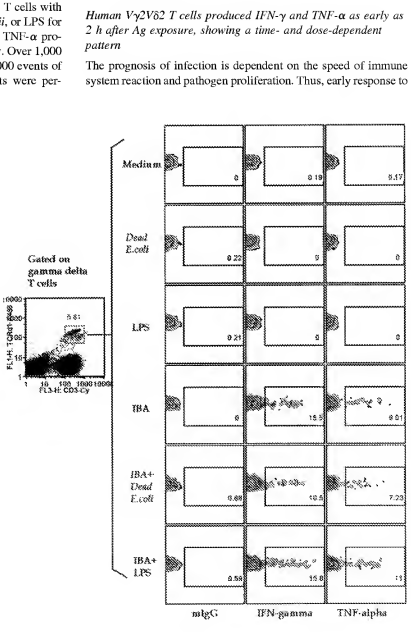

FIGURE 1. Human Vγ2Vδ2 T cells produced IFN-γ and TNF-α in response to the live bacterial product, IBA, but not to dead bacteria and LPS. Human PBMC were cultivated in RPMI 1640 medium containing either IBA (2 mM), heat-killed *E. coli* (5×10^5 CFU/ml), heat-killed *M. morganii* (5×10^5 CFU/ml), or LPS (final concentration of 1 μg/ml) for 18 h, and were then analyzed for intracellular IFN-γ and TNF-α production by γδ T cells using three-color flow cytometry, including an anti-Vδ2 mAb. Over 1,000 Vγ2Vδ2 T cells in each sample, collected from 200,000 events of gated lymphocytes, were analyzed. Human γδ T cells did not produce IFN-γ and TNF-α in response to stimulation by heat-killed *E. coli* or LPS, but over 15% of these cells generated IFN-γ, and 9% generated TNF-α in response to stimulation with 2 mM IBA, a live bacterial product. Neither heat-killed *E. coli* nor LPS augmented the IBA effect, demonstrating that production of IFN-γ and TNF-α was strictly regulated by IBA. Data were representative of three experiments.

live bacterial infection is critical for the immune system to eliminate pathogens. To determine how quickly human γδ T cells could generate IFN-γ and TNF-α after exposure to the live bacterial product, IBA, we assessed intracellular cytokine production at different times after stimulation with 2 mM IBA. About 1% of γδ T cells began to produce IFN-γ and TNF-α as early as 2 h after Ag exposure, and this increased to 6% at 18 h, showing a time-dependent (Fig. 2, *a* and *b*) and dose-dependent response (Fig. 2*c*). A Vγ2Vδ2 T cell clone derived by stimulation with IAA showed a similar dose-dependent response curve to IBA and IAA in production of IL-2 and IFN-γ (Fig. 2, *d* and *e*). In contrast to recently stimulated Vγ2Vδ2 T cells, most Vγ2Vδ2 T cell clones tested in this study lost their capacity to secrete TNF-α (data not shown), suggesting that data interpretation should be cautious when comparing the response of different γδ T cell preparations.

The finding that human γδ T cells produced detectable IFN-γ and TNF-α as early as 2 h after Ag exposure suggests that γδ T cells participate in the early innate immune response. The time- and dose-dependent patterns of γδ T cells in response to the live bacterial products, IBA and IAA, suggest that amount of IFN-γ and TNF-α produced by γδ T cells correlates with numbers of bacteria and infective time. If exposed to IBA for 1 day, γδ T cells did not expand (Fig. 3), but they did produce cytokines (Fig. 1). Continued stimulation for 10 days with IBA, but not with dead bacteria (such as heat-killed *E. coli*, *M. morganii*, or *S. typhii*) and LPS, caused γδ T cell expansion (Fig. 3). This expansion started at day 6 and maximized at days 10–13 (data not shown). In addition, continuous IBA exposure caused γδ T cells to continually produce cytokines, with production peaking at days 3 and 4 (data not shown). These data demonstrate that both cytokine production and cellular expansion of γδ T cells were regulated by the live bacterial product, IBA, and that the immune function of γδ T cells correlates with the dose and duration of Ag exposure.

Human γδ T cells show on/off/on cycling in generation of IFN-γ and TNF-α

Virus-specific $CD8^+$ αβ T cells show a pattern of rapid on/off cycling in cytokine production that is dependent on peptide MHC complexes (25). To determine whether γδ T cells could cease and resume production of IFN-γ and TNF-α dependent on the absence or presence of the live bacterial product, IBA, we washed PBMC after 18 h of IBA stimulation, cultured these IBA-primed PBMC in medium, and analyzed them by intracellular cytokine staining. The number of γδ T cells producing cytokine declined by up to 70% after 2 h and became undetectable by 6 h in the absence of IBA (Fig. 4*a*). When IBA stimulation was restored, γδ T cells resumed

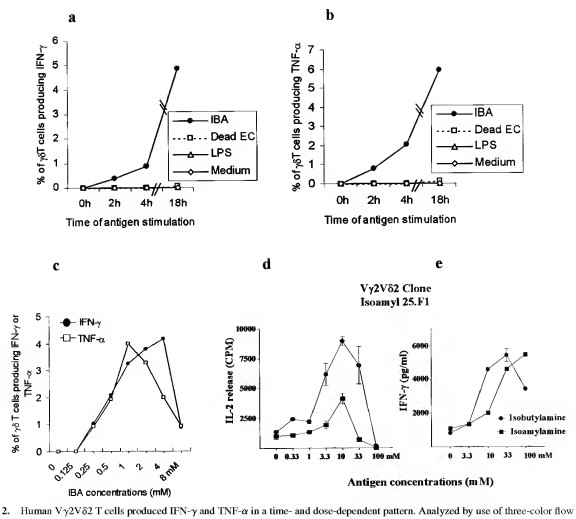

FIGURE 2. Human Vγ2Vδ2 T cells produced IFN-γ and TNF-α in a time- and dose-dependent pattern. Analyzed by use of three-color flow cytometry, γδ T cells started to produce IFN-γ and TNF-α as early as 2 h and increased thereafter in a time- (*a* and *b*) and dose-dependent manner (*c*) when stimulated with 2 mM IBA, a live bacterial product, but not with heat-killed *E. coli* (dead EC, 5×10^5 CFU/ml) or LPS (1 μg/ml). A Vγ2Vδ2 T cell clone showed a similar dose-dependent response curve in response to live bacterial product, IBA and IAA, by producing IL-2 (*d*, HT-2 cell bioassay) and IFN-γ (*e*, ELISA).

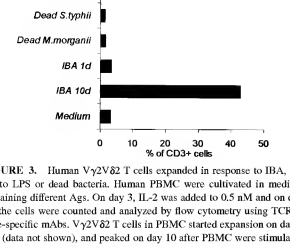

FIGURE 3. Human Vγ2Vδ2 T cells expanded in response to IBA, but not to LPS or dead bacteria. Human PBMC were cultivated in medium containing different Ags. On day 3, IL-2 was added to 0.5 nM and on day 10, the cells were counted and analyzed by flow cytometry using TCR V gene-specific mAbs. Vγ2Vδ2 T cells in PBMC started expansion on day 5 or 6 (data not shown), and peaked on day 10 after PBMC were stimulated for 10 days with IBA, but not with LPS or heat-killed *E. coli*, *S. typhii*, or *M. morganii*. The data were representative of stimulation of γδ T cells with different concentrations of LPS (10 ng to 1 μg) and heat-killed bacteria (1×10^5–1×10^7 CFU). γδ T cells did not expand in PBMC cultures exposed to IBA for 24 h, washed, then cultured in medium for 9 more days (IBA, 1 day).

IFN-γ and TNF-α production (Fig. 4*b*). Vγ2Vδ2 T cell viability after IBA re-exposure did not change significantly. Because production of IFN-γ and TNF-α was abolished by the addition of actinomycin D (data not shown), γδ T cell-mediated production of these cytokines, as in αβ T cells (25), required de novo RNA synthesis. This regulation was specific for γδ T cells because IBA did not directly stimulate αβ T cells, NK cells, NK T cells, or monocytes to produce cytokines. Thus, during a mild bacterial infection, Vγ2Vδ2 T cells do not expand, but they do produce IFN-γ and TNF-α, showing "on/off/on" cycling of cytokine production.

We recapitulated this in vitro cytokine on/off/on cycling of γδ T cells in an in vivo model. Human PBMC pretreated with 1 mM IBA for 18 h resulted in 8% of γδ T cells producing IFN-γ and TNF-α. We then washed the PBMC, immediately reconstituted SCID mice with these PBMC, and simultaneously infected the mice with 5×10^6 CFU of live *E. coli*. Eighteen hours later, human PBMC were collected from the peritoneal lavage of SCID mice for intracellular cytokine staining. The recovery rate of human Vγ2Vδ2 T cells from hu-SCID mice, similar to whole PBMC, was 30–50%. Two-color analysis shows that 6.27 and 10.9% of human γδ T cells from SCID mice infected with the live *E. coli* expressed intracellular IFN-γ and TNF-α, respectively, whereas only 0.61 and 1.43% of γδ T cells from mock-infected mice expressed intracellular IFN-γ and TNF-α, respectively (Fig. 5). These data suggest that γδ T cells cease cytokine production in the absence of stimulation in vivo.

IFN-γ and TNF-α produced by γδ T cells are critical for monocyte-mediated killing of extracellular bacteria

Fresh PBMC containing an average of 20,000 Vγ2Vδ2 T cells that were stimulated with nonpeptide alkylamine Ags secreted up to 700 μg/ml of IFN-γ or TNF-α. IFN-γ and TNF-α are critical for monocyte-mediated killing of extracellular bacteria (14, 15, 18). To test the ability of human γδ T cells to influence monocyte-mediated killing of *E. coli* in vitro, mock-depleted PBMC or those depleted of Vδ2 T cells were cultured in medium containing the Vγ2Vδ2 T cell-specific Ag, IBA, for 4 days. Monocytes from these cultures were then analyzed for their ability to kill *E. coli*. Monocytes from PBMC cultures that were mock depleted of Vδ2 T cells killed up to 3-fold more bacteria than monocytes from the cultures depleted of Vδ2 T cells. Inclusion of neutralizing mAbs to either IFN-γ or TNF-α during the 4-day culture abrogated the Vδ2 T cell-dependent monocyte-mediated killing of *E. coli*, whereas adding back either IFN-γ or TNF-α to the cultures completely restored bacterial killing activity (Fig. 6). These data strongly suggest that IFN-γ and TNF-α produced by human γδ T cells in response to stimulation by the live bacterial product, IBA, are critical for monocyte-mediated killing of extracellular bacteria.

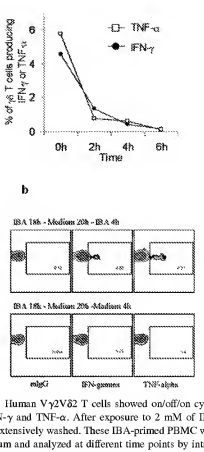

FIGURE 4. Human Vγ2Vδ2 T cells showed on/off/on cycling in generation of IFN-γ and TNF-α. After exposure to 2 mM of IBA for 18 h, PBMC were extensively washed. These IBA-primed PBMC were then cultured in medium and analyzed at different time points by intracellular cytokine staining. Cytokine secretion declined by up to 70% after 2 h, and completely ceased after 6 h in absence of IBA (*a*). When IBA was restored after 20 h in media, γδ T cells resumed IFN-γ and TNF-α production, showing an on/off/on cycling (*b*). There was no significant change in Vγ2Vδ2 T cell viability throughout the time course of this experiment.

Discussion

It is generally assumed that upon stimulation, Vγ2Vδ2 T cells continually secrete cytokines and expand later. However, cytokine production ceases upon disappearance of the live bacterial product, IBA, and resumes when antigenic contact is restored (Fig. 4), showing on/off/on cycling. Vγ2Vδ2 T cells do not expand, but they do produce IFN-γ and TNF-α in response to less than 24 h of stimulation with >125 μM IBA (Figs. 1–3). These data indicate that there exists a time and dose threshold for stimulation of Vγ2Vδ2 T cells by live bacterial products. The significance of this finding has not been appreciated, nor has the underlying mechanism been described. We speculate that Vγ2Vδ2 T cells produce

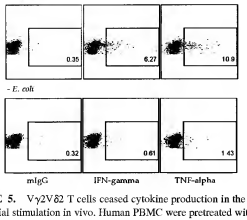

FIGURE 5. Vγ2Vδ2 T cells ceased cytokine production in the absence of bacterial stimulation in vivo. Human PBMC were pretreated with 1 mM IBA for 18 h, and SCID mice were then reconstituted with these PBMC whose IBA had been washed away completely, and were simultaneously infected (or mock infected) i.p. with *E. coli* (5×10^6 CFU/mouse). Eighteen hours later, PBMC were collected from the peritoneal lavage of SCID mice for intracellular cytokine staining. Two-color analysis showed that 6.27 and 10.9% of human γδ T cells from SCID mice infected with *E. coli* expressed intracellular IFN-γ and TNF-α respectively (*top*), whereas only 0.61 and 1.43% of γδ T cells from mock-infected SCID mice secreted those cytokines, respectively.

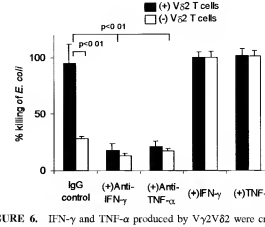

FIGURE 6. IFN-γ and TNF-α produced by Vγ2Vδ2 were critical for monocyte-mediated killing of extracellular bacteria. After depletion or mock depletion of Vγ2Vδ2 T cells, human PBMC (1×10^6 cells) in 1 ml of RPMI 1640 medium containing 1 mM IBA were cultivated in 24-well plates in the presence or absence of anti-IFN-γ (1 μg/ml), anti-TNF-α (1 μg/ml), IFN-γ (100 IU/ml), or TNF-α (100 IU/ml) for 4 days. After three washes to eliminate antibiotics, 0.65 ml of BSS, 50 μl of ice-cold normal human serum, and 0.3 ml of *E. coli* (7.5×10^6 CFU/ml, middle stationary phase) were added to each well. After a 20-min cultivation at 37°C enabling monocytes to ingest bacteria, noningested bacteria were washed away. One plate was taken immediately as time zero, and another plate was taken as time 90 min after incubation at 37°C for bactericide. Bacteria within the monocytes were released by treatment with detergent, diluted serially with water, plated on LB agar, and incubated for CFU counting. Monocytes from PBMC cultures that were mock depleted of Vδ2 T cells killed up to 3-fold more *E. coli* than monocytes from cultures depleted of Vδ2 T cells. Inclusion of neutralizing mAbs to either IFN-γ or TNF-α abrogated the Vδ2 T cell-dependent monocyte-mediated killing of *E. coli*, whereas adding back either IFN-γ or TNF-α to the cultures completely restored monocyte-mediated bacterial killing activity.

cytokines in response to a mild bacterial infection and cease cytokine generation when bacteria are killed; if the infection lasts longer and is more severe, Vγ2Vδ2 T cells continue cytokine production and subsequently expand. These expanded Vγ2Vδ2 T cells may then play an important role in killing infiltrated macrophages to control inflammation (32, 33).

It is well known that IFN-γ and TNF-α are required for monocyte-mediated bactericide, in particular, IFN-γ. These two cytokines work synergistically in host resistance to bacterial infection (34). Because many immune cells produce IFN-γ and TNF-α, depletion of Vδ2 T cells substantially reduces but does not completely deplete the total levels of IFN-γ or TNF-α. In our experiment (Fig. 6), the addition of either exogenous IFN-γ or TNF-α completely restored monocyte-mediated bactericide, reflecting the synergistic effects of exogenous and endogenous TNF-α and IFN-γ.

This in vitro antibacterial effect has been recapitulated in an in vivo hu-SCID model. γδ T cells recovered from *E. coli*-infected, but not uninfected, hu-SCID mice produced IFN-γ and TNF-α (Fig. 5). Hu-SCID mice receiving PBMC that were mock depleted of γδ T cells had significantly higher resistance to bacterial challenge compared with those mice receiving PBMC depleted of γδ T cells. Whereas in vivo expansion of human γδ T cells in reconstituted, infected SCID mice did not occur until 7 days postinfection, the antibacterial effect of human γδ T cells in our studies was evident in as few as 17 h postinfection for *S. aureus* and 27 h for *E. coli*, indicating that expansion of γδ T cells is not required for an antibacterial response. SCID mice reconstituted with mock-depleted human PBMC had higher levels of serum human IFN-γ compared with mice reconstituted with human PBMC depleted of Vδ2 T cells. Further, there was a remarkable inverse correlation between blood IFN-γ levels and *E. coli* CFU in hu-SCID mice (36). Therefore, it is likely that early antibacterial effects result from a cytokine-dependent mechanism.

In contrast, cytokine release can be harmful and sometimes lethal to the host (21–24, 35). Cytokines are responsible for many of the symptoms of viral and bacterial infection. Several cytokines, in particular TNF-α and IFN-γ, have been shown to be responsible for pathological reactions, which may lead to shock and death observed during infection with gram-negative bacteria and in response to endotoxins (20).

The ability to turn cytokines on and off without cell expansion shows the versatility of the cellular immune response and provides a mechanism for maintaining and mediating an effective immune response while reducing immunopathology. As cytokines are rapidly secreted, continued production in the absence of Ag would lead to their inappropriate release in noninfected tissues as activated Vγ2Vδ2 T cells migrated through various organs or the bloodstream. By maintaining cytokine production only upon continued IBA contact, Vγ2Vδ2 T cells minimize the immunopathology that would occur if these cytokines were secreted in a continual manner. Further, by limiting cytokine production to periods of direct contact with infected targets, Vγ2Vδ2 T cells focus their resources at the site of infection.

Vγ2Vδ2 T cells, as an intact functional unit within PBMC, produced cytokines in an on/off/on pattern in response to the live bacterial product, IBA. It is not clear whether the same T cell clones that ceased cytokine production subsequently resumed production on re-exposure to Ag, but regardless, this pattern of cytokine secretion consequently results in an immune response that is tightly controlled by the presence or absence of Ag. Effector memory type T cells can produce cytokines rapidly in response to Ag stimulation, whereas naive or central memory T cells need several signaling steps for activation. It is possible that only certain subpopulations of memory Vγ2Vδ2 T cells produce cytokines in response to IBA stimulation, and further studies are necessary to define these subpopulations.

Time and dose thresholds of Vγ2Vδ2 T cells in response to alkylamines existed not only at high concentrations, but also at physiological concentrations of IBA. Vγ2Vδ2 T cells cultured in low doses of IBA over 13–14 days had up to 2-fold higher cellular expansion in response to a subsequently high dose of IBA stimulation as compared with those cultured in absence of IBA (our unpublished data). Physiologically low concentrations of alkylamine Ags, which may be derived from either plant foodstuffs or from commensal gut bacteria, are ubiquitously found in human body fluids (1). These low concentrations of alkylamines may prime γδ T cells to respond to the higher concentrations of secreted alkylamine Ags associated with a bacterial infection, thereby leading to enhanced bacterial resistance.

Depending on conditions, stimulation of fresh human PBMC with IBA in vitro induced only 2–15% of Vγ2Vδ2 T cells to produce IFN-γ and TNF-α, compared with 3–50% of peptide-specific αβ T cells (25). However, these γδ cells are much more efficient at cytokine production on a per cell basis than αβ T cells (19). Further, these Vγ2Vδ2 T cells, reactive to nonpeptide Ags, are 5- to 50-fold more numerous than αβ T cells specific for any one peptide, and may thus have a major physiologic impact.

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